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Mutant Analysis by Rescue Gene Excision: a technique for mosaic studies in *Drosophila*

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Abstract

A host of classical and molecular genetic tools make *Drosophila* a tremendous model for the dissection of gene activity. In particular, the FLP-FRT technique for mitotic recombination has greatly enhanced gene loss-of-function analysis. This technique efficiently induces formation of homozygous mutant clones in tissues of heterozygous organisms. However, the dependence of the FLP-FRT method on cell division and other constraints also impose limits on its effectiveness. We describe here the generation and testing of tools for Mutant Analysis by Rescue Gene Excision (MARGE), an approach whereby mutant cells are formed by loss of a rescue transgene in a homozygous mutant organism. Rescue-transgene loss can be induced in any tissue or cell type and at any time during development or the lifetime of the adult by using available *FLP*, *Gal4* and *Gal80^{ts}* reagents. The simultaneous loss of a constitutive fluorescence marker (GFP or RFP) identifies the mutant cells. We demonstrate the efficacy of the MARGE technique by flip-out (clonal and disc-wide) of a *Ubi-GFP*-carrying construct in imaginal discs, and by inducing a known *yki* mutant phenotype in the *Drosophila* ovary.

Keywords

MARGE; Flip-out Rescue Cassette vectors; FoRC vectors; CKO; conditional knockout; clonal analysis; yorkie

Drosophila melanogaster is a powerful genetic model thanks to a plethora of classical and molecular genetic tools developed over many decades of groundbreaking research. In particular, the development of the FLP-FRT technique for mitotic recombination revolutionized the generation and use of mosaic animals. This method facilitated once laborious investigations, such as the study of later functions of early lethal loci or the assessment of cell autonomy/non-autonomy of a genetic trait (Xu and Rubin, 1993; Xu and Rubin, 2012). In the FLP-FRT method, the Flip recombinase, or Flippase, promotes DNA

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exchange between homologous chromosomes by mediating an inter-chromosomal recombination event at FRT sequences located near the centromeres (Fig. 1A). When this occurs in heterozygous cells (+/−) and during mitosis, chromosome segregation can result in the formation of two daughter cells homozygous (+/+ and −/−) for the exchanged region (Fig. 1A). Although this provides a highly efficient method to generate homozygous mutant clones, the FLP-FRT technique's dependence on cell division limits its usefulness to certain tissues and developmental stages. In addition, the technique cannot be used for genes on the fourth chromosome or any gene located between the FRT and the centromere.

Unlike the FLP-FRT method, the conditional knockout (CKO) technique first developed in mouse is based on an intra-chromosomal recombination event and is therefore independent of mitosis. Hence, one avenue for improvement of mosaic analysis in *Drosophila* lies in developing an approach similarly based on an intra-chromosomal reaction. Three CKO-like approaches have been reported in fly (Ou H and Lei T., 2013; Frickenhaus et al., 2015; Jin et al., 2016). In these studies, FRT-, LoxP- or att-bound mutant alleles were generated by modification of either endogenous or transgenic genomic loci. However, all three approaches require advanced molecular and/or genetic techniques to generate the required reagents and mutant cells can only be identified if antibodies against the gene product are available (Ou H and Lei T., 2013; Frickenhaus et al., 2015; Jin et al., 2016).

Here, we describe a simple and precise strategy to efficiently induce differentially-marked mutant tissue applying an approach that we call MARGE, for *Mutant Analysis by Rescue Gene Excision*. MARGE is based on the FLP-mediated excision of FRT-flanked DNA from the genome (Golic and Lindquist, 1989). An FRT-flanked cassette carrying a rescuing transgene and a constitutive fluorescent marker is excised in a homozygous mutant background leading to the simultaneous loss of rescue and fluorescence (Fig. 1B). Thus, mutant cells are readily identifiable amid surrounding (rescued) wild type cells that continue to express the constitutive marker. We have designed a series of Flip-out Rescue Cassette (FoRC) vectors for P-element (P) or attB (attB) transgenesis that carry one of two constitutive fluorescent markers, *Ubi-GFP* (G) or *Ubi-RFP* (R), and a multiple cloning site (m) or a Gateway attP1/2 site (a), altogether flanked by double FRT sequences (Fig. 1C). The FoRC vectors are thus compatible with traditional, Gateway, and recombineering approaches to cloning and with random (P element) or site-directed (attB) transgenesis. Insertion of a rescuing DNA fragment, either genomic DNA or a minigene (e.g. promoter-cDNA), into the cloning site generates a construct suitable for use in the MARGE technique.

In the CKO tradition, MARGE is based on an intra-chromosomal recombination event and is thus efficient and proliferation-independent. Loss of gene function can be induced in any tissue and at any time in the organism's life cycle. Since the pattern of expression of the Flippase determines which cells are affected, the MARGE approach offers numerous potential applications using already available FLP reagents. These include heat-shock induced (*hs-FLP*) and tissue specific (e.g. *ey-FLP* and *Ubx-FLP*) Flippases and a vast choice of *GAL4-driver UAS-FLP* combinations; temporal control can be imposed in the latter case by inclusion of a *tub-Gal80^{ts}* transgene. Utilizing some of these tools, we provide examples of clonal excisions of a *FoRC.PGm* cassette (GFP loss) by *hs-FLP* in eye-antennal and wing

discs (Fig. 2A–B'), as well as an example of disc-wide excision by *ey-FLP* in the eye-antennal disc (Fig. 2A'').

Experimental conditions that can be readily investigated with MARGE, but not with the traditional FLP-FRT technique, are numerous. They include loss-of-function targeting transiently quiescent cells (e.g. cells of L1 imaginal discs), cells that have undergone their final division (e.g. differentiating cells at larval or pupal stages, or fully differentiated cells of the adult fly), syncytia (e.g. muscle tissue), and all cells of a specific type within a tissue or organ. In addition, loss-of-function can be readily coupled to exogenous protein expression or combined to analyze multiple loci simultaneously. In addition, the MARGE technique is applicable to any gene, including genes located near centromeres or on the fourth chromosome.

To test the MARGE approach, we chose to reproduce a known loss-of-function phenotype of the gene *yorkie* (*yki*). The Yki transcriptional co-activator (homologue of the vertebrate YAP and TAZ proteins) is a regulator of proliferation and survival in most cell types and across the Metazoa. It is therefore not surprising that *yki* mutant alleles are embryonic lethal. Nonetheless, FLP-FRT-based studies of *yki* mutant clones in many *Drosophila* tissues have provided ample evidence of its function in cell proliferation and survival (Huang et al., 2005; Zhang et al., 2009). In the follicle cells of the *Drosophila* ovary, Yki is active specifically at the early stages of egg chamber development (stages 1–6) when robust proliferation is necessary in order to produce a sufficient number of follicle cells (Fig. 3A) (Meignin et al., 2007; Polesello and Tapon, 2007). At these stages, Yki activity is critical for the expression of the transcription factor Cut throughout the follicular epithelium (Fig. 3A'; Polesello and Tapon, 2007; Koontz et al., 2013). Hence, in *yki^{B5}/yki^{B5}* mutant clones, Cut expression is strongly reduced or lost (Koontz et al., 2013). To reproduce this phenotype, we introduced the *FoRC.PGm-yki⁺* rescue construct (Fig. 3B) into a *yki^{B5}/yki^{B5}* mutant background. We then induced excision of the *Ubi-GFP yki⁺* cassette by *hs-FLP* in wild type (*+/+*) and homozygous mutant (*yki^{B5}/yki^{B5}*) females. Dissection and immunostaining with anti-GFP and anti-Cut antibodies revealed the presence of GFP-negative clones in the follicular epithelia of both *+/+* or *yki^{B5}/yki^{B5}* ovaries (Fig. 3C and D); loss of Cut was observed in GFP-negative *yki^{B5}/yki^{B5}* clones (Fig. 3D') but not GFP-negative *+/+* clones (Fig. 3C'). Thus, loss of the *FoRC.PGm-yki⁺* rescue construct in the homozygous mutant resulted in the expected *yki* loss-of-function phenotype.

In conclusion, we have presented a conditional mutant approach, MARGE, that both facilitates and increases the versatility of loss-of-function analysis at all stages of *Drosophila* development and in the adult.

Methods

The DNA, as well as map and sequence information, for all five *FoRC* vectors is available from Addgene: the *yki* genomic sequence used in the *FoRC.PGm-yki⁺* construct was amplified from *w*; *Canton-S* with the primers *yki*-F (5'-AACCTCAGCTTTACATTTGTGTCATGCGACATTAGTTATTG-3') and *yki*-R (5'-TTGGCCGGCCGAGAGATGAATTTGACGCAAGAGAATC-3'); cloning strategy for all

DNAs is available upon request. The *FoRC.PGm-yki⁺* transgenic line was generated by injection into *w¹¹¹⁸* embryos (BestGene). The Flippases can be obtained from the Bloomington *Drosophila* Stock Center (BDSC): *ey-FLP* BDSC #5580 (<http://flybase.org/reports/FBti0015982.html>) and *hs-FLP122* BDSC #23649 (<http://flybase.org/reports/FBtp0001101.html>). The *yki^{B5}* allele (Huang et al., 2005) was kindly provided by K. Irvine. To test for excision of the *FoRC* cassette, the *FoRC.PGm-yki⁺* flies were crossed to *ey-FLP* or *hs-FL* flies; first instar (L1) *hs-FLP*, *FoRC.PGm-yki⁺* larvae were heat shocked (37°C) for 1 hr. Imaginal discs from *hs-FLP*, *FoRC.PGm-yki⁺* or *ey-FLP*, *FoRC.PGm-yki⁺* L3 larvae were stained with rabbit anti-GFP (1:10,000; Invitrogen). To test the MARGE approach, we used the technique to generate *yki^{B5}/yki^{B5}* mutant clones in the ovary. Rescued *yki^{B5}/yki^{B5}*; *FoRC.PGm-yki⁺*/*TM6B* males were crossed to *hs-FLP/X*; *yki^{B5}*; 3/*SM6::TM6B* females; 3–4 day old female progeny homozygous for *yki^{B5}* (*hs-FLP/X*; *yki^{B5}/yki^{B5}*; *FoRC.PGm-yki⁺*/3) were heat shocked for 1 hr on two consecutive days and their ovaries were dissected 5 days later to be processed for immunostaining. Wild type controls (*hs-FLP/X*; *FoRC.PGm-yki⁺*/3) were treated identically. Antibodies were from the Developmental Studies Hybridoma Bank (DSHB) unless otherwise noted: mouse anti-Cut (1:80), rat anti-DE-cad (1:50), and rabbit anti-GFP (1:10,000; Invitrogen). ALEXA-488-, Cy3- or Cy5-conjugated goat secondary antibodies (anti-rabbit, anti-mouse and anti-rat; Jackson ImmunoResearch Laboratories) were used at 1:250. Images were collected on a Leica DM5500 Q confocal microscope and were processed for publication with Adobe Photoshop (CS4).

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Figure 1. Conditional loss-of-function in *Drosophila* by MARGE

The MARGE approach, unlike the FLP-FRT technique, does not depend on proliferation (A–B). (A) Diagram of the established FLP-FRT technique. Following mitosis, recombined homologous chromosomes from heterozygous mother cells can segregate to yield pairs of daughter cells either homozygous (50% outcome; shown) or heterozygous (50% outcome; not shown) for the exchanged DNA region. (B) Diagram of the MARGE approach. A transgene carrying a wild type locus or a rescuing mini-gene is introduced into the genome and is used to rescue the phenotype caused by mutation of the endogenous locus (*yki* is used as an example). Excision of the rescuing transgene by Flippase restores the homozygous mutant condition, and concomitantly removes the fluorescent marker. (C) Schematics of the series of Flip-out Recombination Cassette (FoRC) vectors designed for this study. The nomenclature, following the FoRC, corresponds to transgenesis method (P = P element, attB = site-directed), marker (G = GFP, R = RFP) and cloning site (m = multiple cloning site, a = Gateway attP1+P2). Gateway constructs contain the *ccdB* gene, which is excised upon successful integration of the rescuing DNA. All FoRC vectors are Amp^R (CaSpeR-2 based)

and the “m” versions can be easily modified for the cloning of large genes by recombineering. The unique restriction sites of the MCSs are shown below.

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Figure 2. Clonal and disc-wide FLP-mediated excision of a *FoRC.PGm* cassette

All panels show L3 eye-antennal (A–A'') and wing (B–B') imaginal discs from *FoRC.PGm-yki+* transgenic flies, wild type at the endogenous *yki* locus, immunostained for GFP. The transgene carries the *Ubi-GFP* marker and *yki+* genomic DNA. The presence of extra copies of *yki+* does not provide a marked growth advantage to GFP-positive cells, and homozygous *FoRC.PGm-yki+* flies with 4 copies of *yki+* are of normal size; this is most likely due to the use of the endogenous *yki* promoter which is likely autoregulatory (Slattery et al., 2014). (A,B) In the absence of Flippase, GFP marks all cells. (A',B') *hs-FLP* induces clonal loss of *Ubi-GFP*, GFP marks cells in which the *FoRC* cassette is still present. (A'') *ey-FLP* induced disc-wide loss of *Ubi-GFP*; immunostaining superimposed on DIC image is shown to the right. Cells of the optic stalk and some cells on the antennal side do not express *ey-FLP* and thus retain the *FoRC* cassette. GFP-positive cells at the bottom left are non-disc, loose cells caught in the disc fold.

Figure 3. MARGE-induced mutant clones recapitulate the loss of Cut expression in *yki* mutant follicle cells

MARGE-induced *yki*^{B5}/*yki*^{B5} mutant cells show loss of Cut expression. (A–A') Wild type ovariole from a *w*; Canton-S female. (A) Staining for E-cadherin (anti-DE-cad, magenta) and DNA (DAPI, blue) to visualize the egg chambers; stages represented in this ovariole are marked (2–5 and 7). (A') Immunostaining for Cut (anti-Cut, red) reveals presence of the protein in the follicle epithelium at the Cut-expressing stages 2 through 5, but not at stage 7. (B) Map of the *FoRC.PGm-yki*⁺ flip-out cassette carrying the *Ubi-GFP* marker and the *yki*⁺ genomic rescue fragment. (C–C') A *hs-FLP/X*; *FoRC.PGm-yki*^{+/3} egg chamber (stage 5/6) stained for GFP (anti-GFP, green), E-cadherin (anti-DE-cad, magenta), Cut (anti-Cut, red), and DNA (DAPI, blue). After heat-shock, GFP staining (green) reveals the mosaicism for the *FoRC.PGm-yki*⁺ cassette in the follicular epithelium (C), but Cut expression (C') is normal due to endogenous Yki. (D–D') A *hs-FLP/X*; *FoRC.PGm-yki*^{+/3} egg chamber (Stage 5) homozygous mutant at the *yki* locus (*yki*^{B5}/*yki*^{B5}) and stained for GFP (anti-GFP, green), E-cadherin (anti-DE-cad, magenta), Cut (anti-Cut, red), and DNA (DAPI, blue).

After heat-shock, loss of the *FoRC.PGm-yki+* cassette induces GFP-negative *yki^{B5}*-mutant clones (yellow arrows) in the follicular epithelium; Cut expression is lost in the *yki^{B5}/yki^{B5}* mutant cells (D').

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